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# Mechanisms of Non-Opsonized Zymosan-Induced and Luminol-Enhanced Chemiluminescence in Whole Blood and Isolated Phagocytes

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**Summary:** A luminol-dependent non-opsonized zymosan-induced chemiluminescence method for phagocytes in small quantities of whole blood (40 µl; final dilution: 1 : 14) is described. It was characterized with reference to cellular and humoral components, and also applied to isolated neutrophils, eosinophils and monocytes. Normal values for whole blood chemiluminescence and for neutrophils, eosinophils and monocytes are presented. From the chemiluminescence characteristic of distinct phagocytes and their frequency distribution pattern in whole blood, it is concluded that whole blood chemiluminescence has its source predominantly in neutrophils. The question as to the origin of chemiluminescence in phagocytes of whole blood and isolated neutrophils is investigated. The results support the importance of the myeloperoxidase-$H_2O_2$-halide system, but also go beyond this. The release of arachidonic acid by phospholipase $A_2$ and of diacylglycerol and inositol trisphosphate by phospholipase C, the metabolism of arachidonic acid by the cyclooxygenase and lipoxygenase pathway, the activation of membrane NADPH oxidase by diacylglycerol and the calcium mobilisation by inositol trisphosphate are necessary for the chemiluminescence reaction. Inhibition of either mechanism suppresses the chemiluminescence response. The interaction of non-opsonized zymosan with plasma opsonins, phagocyte Fc- and complement receptors, respectively, for the initiation of chemiluminescence, was investigated. Non-opsonized zymosan initiates a chemiluminescence response in blood phagocytes in the absence of opsonin from the interaction of the zymosan polysaccharide component glucan with the complement receptor type 3. In the presence of plasma this receptor type also mediates the major chemiluminescence response brought about by the zymosan-coated cleavage products of complement fraction three, iC3b and to a minor degree C3b, while immunoglobulin G-coated zymosan interaction with the Fc-receptor is in this case of minor importance.

## Introduction

Upon interaction with particulate stimuli or chemical stimulation, polymorphonuclear leukocytes (neutrophilic and eosinophilic granulocytes), monocytes and macrophages respond with a chain of biochemical and cytophysiological events commonly known as the respiratory burst.

This respiratory burst is characterized by an increase in glucose dehydrogenation via the hexose monophosphate shunt and a non-mitochondrial increase in oxygen consumption. By enzymatic and non-enzymatic reactions, oxygen is largely converted into superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and probably hydroxyl radical (· OH) and singlet oxygen ($^1O_2$) (1). These excited oxygen species react with certain biological substrates to become electronically excited products which generate light upon relaxation. The relatively poor quantum yield of this native chemiluminescence can be increased more than a thousand fold by using cyclic hydrazides such as luminol as the bystander substrate.

Many investigators have employed luminol-enhanced chemiluminescence of isolated polymorphonuclear leukocytes to measure their oxygenation capacity or,

with a constant granulocyte concentration and variable amounts of plasma, the opsonic capacity of plasma (2).

The high sensitivity of chemiluminogenic probing, however, allows measurement of phagocyte oxygenation activity using diluted native whole blood (see l.c. (1, 2) for examples). The numerous cellular and humoral components which modulate and contribute to the chemiluminescence response of whole blood, have not, however, been well defined. In the first part of our work the influence of plasma and of erythrocytes as well as the contribution of phagocytes other than neutrophilic granulocytes were investigated separately. Normal values for chemiluminescence in whole blood and isolated blood cells (neutrophils, eosinophils, monocytes) were established.

The second part of our work contributes to the ongoing controversy concerning the origin of zymosan-induced luminol-dependent chemiluminescence.

The myeloperoxidase $H_2O_2$-halide system, seen as responsible in the generation of singlet oxygen and hypochlorous acid for chemiluminescence in activated neutrophils (3) was recently challanged by *Cheung* et al. (4) who postulated that the metabolism of arachidonic acid via the lipoxygenase and cyclooxygenase pathway is the source of chemiluminescence.

We investigated the influence of supplemented superoxide dismutase, catalase and myeloperoxidase, of metabolic inhibitors and of scavengers of reactive oxygen species on chemiluminescence in whole blood and isolated neutrophils.

It was concluded that the interaction between the NADPH oxidase system and arachidonic acid metabolism generates chemiluminescence but that each system alone does not.

In the last part of our study we examined the importance of ligand-receptor-mediated initiation of chemiluminescence in phagocytes.

Efficient interaction between phagocytes and particles requires the participation of opsonins, serum proteins such as immunoglobulin G, and the opsonic fragments of complement factor three (C3) which become attached to the surface of microorganisms or other particles and interact with specific receptors on phagocytic cells (5, 6). For zymosan there are conflicting results regarding its interaction with certain cell types, even in the absence of opsonins (5, 7).

We investigated the initiation of chemiluminescence in whole blood and isolated neutrophils with opsonin-coated zymosan and non-opsonized zymosan as well as the influence of chemicals and antibodies directed against specific opsonins or against distinct complement receptor types on the phagocyte membrane. Our results show that all blood cell phagocytes can interact with zymosan via chemiluminescence response without any opsonin. From this study and the published evidence available, a model suggesting the origin of non-opsonized zymosan-induced and luminol-enhanced chemiluminescence in whole blood and neutrophils is presented.

## Materials and Methods

### Chemicals, media and reagents

Sigma Chemicals Co., Louis, USA: Zymosan A from *Saccharomyces cerevisiae*; N-formyl-1-*L*-methionyl-*L*-leucyl-*L*-phenylalanine (f-Met-Leu-Phe); catalase (EC 1.11.1.6) from bovine liver (2890 U/mg); nordihydroguaiaretic acid; indometacin; benzoic acid (sodium salt); dimethyl sulphoxide; quinacrine; arachidonic acid from porcine liver; dibutyryl cAMP; theophylline; N-acetyl-*D*-glucosamine; human immunoglobulin G; anti-human complement $C_3$; anti-human immunoglobulin G (whole molecule); prostaglandin $E_1$; prostaglandin $E_2$.

Boehringer Mannheim GmbH, FRG: Luminol (5-amino-2.3-dihydro-1.4-phthalazinedione); phosphate-buffered saline *Dulbecco* without $Ca^{2+}$ and $Mg^{2+}$; Minimal Essential Medium *Dulbecco* for chemiluminescence with N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES), without phenol red, without glutamine; superoxide dismutase (EC 1.15.1.1) from bovine erythrocytes (5000 U/mg).

Bayer, Leverkusen, FRG: Chlorpromazine (Megaphen®).

E. Merck, Darmstadt, FRG: *L*-alanine; *D*-mannitol; *L*-histidine; ethylenediaminetetraacetic acid (EDTA); sodium azide; sodium chloride; triethylamine; *Türk*'s solution; *May Grünwald* stain; *Giemsa* stain; sodium fluoride. Myeloperoxidase (EC 1.11.1.7) was donated by Dr. *S. Neumann*.

Pharmacia Fine Chemicals, Sweden: Percoll® (Polyvinylpyrolidone-coated silica gel) for density gradient centrifugation.

Fresenius, Bad Homburg, FRG: Sodium citrate solution (31.3 g/l).

Lumac, Düsseldorf, FRG: Polystyrol chemiluminescence vials (12 mm × 47 mm).

Gödecke AG, Berlin, FRG: Thrombofix solution.

Ortho Diagnostic Systems, Neckargemünd, FRG: OKM 1 monoclonal antibody. Anti $C_{3b}$ (monoclonal) was donated by Dr. *A.-M. Gundermann*.

### Blood

Venous blood, anticoagulated with sodium citrate (9 vol blood + 1 vol sodium citrate) was obtained from healthy blood donors from the local blood bank. To avoid day time dependence on respiratory burst activity of phagocytic blood cells (8), blood was taken between 8.30 and 9.30 a.m.

### Plasma

An aliquot of the venous blood was removed and centrifuged at 12 000 *g* for 2 min on an Eppendorf centrifuge 3200 (Netheler & Hinz, Hamburg) at room temperature.

## Haemoglobin

Haemoglobin was determined in whole blood and isolated erythrocytes as cyanmethaemoglobin (9).

## Isolation of blood cells

### *Polymorphonuclear cells*

A two-step discontinuous gradient was established with 4 ml each of the following two solutions: stock isoosmotic Percoll diluted with sodium chloride (0.15 mol/l) to $\rho = 1.0945$ kg/l and $\rho = 1.0779$ kg/l (10). Blood (up to 4 ml) was applied to the top of the gradient and after centrifugation (350 *g*, 25 min, 20 °C, Minifuge GL with a swing-out rotor, Heraeus Christ), polymorphonuclear cells were separated from the interface of the two density concentrations. Contaminating erythrocytes were lysed by hypotonicity. Polymorphonuclear cells were washed twice in phosphate-buffered saline and adjusted to $1-2 \times 10^6$ cells per ml in Minimal Essential Medium according to staining with *Turk*'s solution. Purity was 98–100%.

### *Erythrocytes*

Erythrocytes were banded at the bottom on the two-step discontinuous Percoll gradient. They were recovered and washed once in phosphate buffered saline. As the density of erythrocytes and polymorphonuclear cells overlap (10), the erythrocytes were again centrifuged on 4 ml Percoll with $\rho = 1.0945$ kg/l. After this the erythrocytes were washed twice in phosphate-buffered saline and resuspended in Minimal Essential Medium to a haematocrit of 0.50. The erythrocytes were essentially free of polymorphonuclear cells.

### *Mononuclear cells and monocytes*

Blood cells, depleted from platelet-rich plasma, were centrifuged on the two-step discontinuous Percoll gradient described for polymorphonuclear cells. Mononuclear cells were harvested from the top of the upper layer, washed twice in phosphate-buffered saline and resuspended in Minimal Essential Medium. Monocytes were identified by esterase staining. The fraction of monocytes in the mononuclear cell suspension ranged from 0.17 to 0.26 ($\bar{x} \pm$ SD: $0.23 \pm 0.03$). The monocyte concentration was adjusted to $1-2 \times 10^6$/ml in Minimal Essential Medium irrespective of the total mononuclear concentration. It has been demonstrated that the chemiluminescence activity of mononuclear cells is due to the monocytes and not to lymphocytes (11). Contamination with polymorphonuclear cells was less than 1%.

### *Eosinophils*

Human eosinophils are denser than neutrophils, but the range of densities of the two cell types overlap. However, if neutrophils are exposed to the chemotactic peptide f-Met-Leu-Phe, which does not stimulate eosinophils, the neutrophil density decreases, shifting them away from the density of eosinophils (12).

Whole blood (4 ml), which had been exposed for 15 min at 37 °C to the chemotactic peptide f-Met-Leu-Phe at $10^{-6}$ mol/l, was added to a three step discontinuous gradient of each 3 ml stock isoosmotic Percoll diluted with sodium chloride (0.15 mol/l) to $\rho = 1.0945$ kg/l, 1.0808 kg/l and 1.0779 kg/l and processed as described in the section "polymorphonuclear cells".

The cell layer between the interface of both lower density concentrations was harvested, washed twice in phosphate-buffered saline and the cell count adjusted to $1-2 \times 10^6$/ml in Minimal Essential Medium. After *Pappenheim* staining, purity was 77%.

## Chemiluminescence

Chemiluminescence assays in whole blood and isolated blood cells were performed on a Biolumat 9505 with a six channel device interconnected with an Apple IIe, a floppy disk II, a video display and an Epson MX 82F/T printer (Laboratorium Prof. Dr. Berthold, Wildbad, FRG) in polystyrene chemiluminescence vials at 37 °C.

## Standard assay

### *Luminol*

Luminol (22.6 mmol/l) was prepared daily in Minimal Essential Medium containing triethylamine (40 mmol/l). Luminol final assay concentration: 0.4 mmol/l.

### *Non-opsonized zymosan*

Zymosan particles were washed twice in phosphate buffered saline, resuspended in Minimal Essential Medium to 100 g/l and stored frozen at −70 °C in small aliquots. Zymosan final assay concentration: 3.5 g/l.

*Pipetting scheme see page 768*

## Modification of the chemiluminescence standard assay

In order firstly to optimize the assay and secondly to study the humoral and cellular aspects of whole blood and isolated cell chemiluminescence, the following modifications and additions were made.

### *Luminol*

The final assay concentration of luminol ranged from 0.04 mmol/l to 2 mmol/l. Various volumes of luminol were balanced with different volumes of Minimal Essential Medium. Whole blood volume (40 µl) and non-opsonized zymosan (20 µl) were constant.

### *Zymosan*

The final assay concentration of non-opsonized zymosan ranged from 0.35 g/l to 35 g/l. Various volumes of non-opsonized zymosan were balanced with different volumes of Minimal Essential Medium. Whole blood volume (40 µl) and luminol (10 µl) were constant.

In some experiments (see tab. 4) opsonized zymosan was used. Zymosan, washed with phosphate-buffered saline (100 g/l), was incubated with normal human pooled plasma at 37 °C for 30 min, washed twice in phosphate-buffered saline, resuspended in Minimal Essential Medium to 100 g/l and stored frozen at −70 °C in small aliquots. Opsonized zymosan final assay concentration: 3.5 g/l.

### *Red blood cells*

Various quantities of red blood cells were added in a constant volume of 20 µl Minimal Essential Medium. Expressed as final assay haemoglobin concentration, the concentration range was from 0.25 g/l to 5.5 g/l (fig. 1). Luminol (10 µl), plasma (20 µl), neutrophil number ($0.5 \times 10^5$) and non-opsonized zymosan (20 µl) were constant.

### *Plasma volume*

Whole blood chemiluminescence was performed with various volumes of plasma (fig. 2). Plasma volume varied in the range

*Pipetting scheme*

Chemiluminescence reaction mixtures were prepared as follows (volume in µl) for
a. whole blood and
b. isolated blood cells.

a. *Whole blood*

| | channel 1/2 'stimulation 1' (CL 1) | channel 3 blank 1 (K 1) |
|---|---|---|
| Minimal Essential Medium | 500 | 520 |
| Luminol | 10 | 10 |
| Blood | 40 | 40 |
| | 5 min incubation 37 °C | |
| Non-opsonized zymosan | 20 | — |

b. *Granulocytes, eosinophils, monocytes*

| | channel 1/2 'stimulation 1' (CL 1) | channel 3/4 'stimulation 2' (CL 2) | channel 5 blank 1 (K 1) | channel 6 blank 2 (K 2) |
|---|---|---|---|---|
| Minimal Essential Medium | 500 | 520 | 520 | 540 |
| Luminol | 10 | 10 | 10 | 10 |
| Cell suspension | 20 | 20 | 20 | 20 |
| Autologous plasma | 20 | — | 20 | — |
| | 5 min incubation 37 °C | | | |
| Non-opsonized zymosan | 20 | 20 | — | — |

Chemiluminescence was continuously recorded until it demonstrated a definite decline. Two chemiluminescence parameters were calculated from the measurements:

(i) peak maximum counts/min values of stimulation reaction. Whole blood: counts/min × 2 × $10^5$ granulocytes; isolated cells: counts/min × 0.5 × $10^5$ cells.

(ii) peak time values (time in min after starting the Biolumat required to reach the peak maximum).

15 µl to 50 µl and was balanced with different volumes of Minimal Essential Medium. Luminol (10 µl), neutrophils (0.5 × $10^5$), red blood cells (20 µl) and non-opsonized zymosan (20 µl) were constant.

*Red blood cells and plasma volume*

Various combinations of red blood cells and plasma in Minimal Essential Medium (from 5 µl red blood cells + 35 µl plasma to 30 µl red blood cells + 10 µl plasma) were added in a constant volume of 40 µl (fig. 3). Luminol (10 µl), neutrophils (0.5 × $10^5$) and non-opsonized zymosan (20 µl) were constant.

*Granulocytes*

Additional granulocytes in Minimal Essential Medium (from 5 × $10^3$ to 5 × $10^5$ cells) were added to whole blood (fig. 4). Various added volumes were balanced with different volumes of Minimal Essential Medium. Luminol (10 µl), whole blood (40 µl) and non-opsonized zymosan (20 µl) were constant.

*Plasma composition*

Two experiments were performed to investigate the role of plasma in whole blood chemiluminescence and in chemiluminescence of isolated neutrophils. Methodological procedure and results are presented in table 1 and table 2.

### Addition of superoxide dismutase, myeloperoxidase and catalase

Superoxide dismutase, myeloperoxidase and catalase were dissolved in and diluted with Minimal Essential Medium to give final assay catalytic activity concentrations of 400 kU/l, 200 U/l and 4800 kU/l, respectively. Experiments were performed with whole blood and isolated neutrophils. Whole blood experiments included the standard assay procedure with non-opsonized zymosan and also opsonized zymosan and plasma-depleted whole blood (washing three times) with opsonized zymosan. Experiments with neutrophils followed the same scheme. All enzymes were incubated with whole blood or neutrophils, in the presence of luminol in Minimal Essential Medium for 5 min at 37 °C; reaction was initiated with non-opsonized or opsonized zymosan. Results are expressed as the quotient of chemiluminescence peak maximum values (CL 1 − K 1) in the presence and in the absence of enzymes (tab. 4).

### Addition of metabolic inhibitors and scavengers of reactive oxygen species

Nordihydroguaiaretic acid was dissolved in dimethylsulphoxide to 10 mmol/l and further diluted in Minimal Essential Medium. Final assay concentration: 0.1 mmol/l. Dimethylsulphoxide has been reported to be a hydroxyl radical scavenger (4). Our control experiments showed, however, that the amount of dimethylsulphoxide we used did not affect the magnitude of the

chemiluminescence response. Arachidonic acid and prostaglandin $E_1$ and $E_2$ were dissolved in ethanol to 30.6 mmol/l and 2.8 mmol/l, respectively, and further diluted in Minimal Essential Medium. Final assay concentration: 0.05 mmol/l and 0.03 mmol/l, respectively. All other drugs were dissolved in Minimal Essential Medium. Final assay concentration in brackets: *L*-alanine (10.5 mmol/l); *D*-mannitol (40 mmol/l); sodium benzoate (1.5 mmol/l); *L*-histidine (21 mmol/l); sodium azide (1 mmol/l); cyanide (1 mmol/l); indometacin (1 mmol/l); quinacrine (1 mmol/l); dibutyryl cAMP (1 mmol/l); theophylline (2 mmol/l); NaF (20 mmol/l); chlorpromazine (0.1 mmol/l).

All drugs were incubated with whole blood or neutrophils in the presence of luminol in Minimal Essential Medium for 10 min at 37 °C; reaction was initiated with non-opsonized zymosan. Results are expressed as the quotient of chemiluminescence peak maximum values (CL 1 – K 1) in the presence and in the absence of inhibitors or scavengers (tab. 5).

### Receptor studies

N-acetyl-*D*-glucosamine (200 mmol/l) and EDTA (3 mmol/l) were incubated with neutrophils or whole blood for 10 min at 37 °C before the start with non-opsonized zymosan according to the standard procedure. Autologous plasma (20 µl) or immunoglobulin G solution (20 µl of 11 g/l) are the source of particular opsonins in the experiments with isolated neutrophils. Among opsonins, complement $C_3$, complement $C_{3b}$ and immunoglobulin G were inhibited with anti-human complement $C_3$, anti-human complement $C_{3b}$ and anti-human immunoglobulin G (whole molecule), respectively, and with a combination of anti-immunoglobulin G and anti-complement $C_3$. Appropriate dilutions of plasma and immunoglobulin G solution with antibodies were prepared according to the titre, as given by the manufacturer. The monoclonal antibody OKM 1 final assay concentration was 5 mg/l.

## Results

### Optimization of whole blood chemiluminescence; reference values

#### *Luminol*

With different luminol concentrations peak maximum is reached between 0.3 to 0.5 mmol/l final assay concentration (not shown). In further experiments a luminol final assay concentration of 0.4 mmol/l was used.

#### *Non-opsonized zymosan*

A steady increase of photon emission was observed with increasing zymosan particle concentration (not shown). In further experiments a non-opsonized zymosan final assay concentration of 3.5 g/l was used.

#### *Erythrocytes*

Figure 1 gives the relationship between increasing numbers of erythrocytes (g haemoglobin per l final assay) and constant numbers of granulocytes ($0.5 \times 10^5$) and constant plasma volume fraction (20 µl).

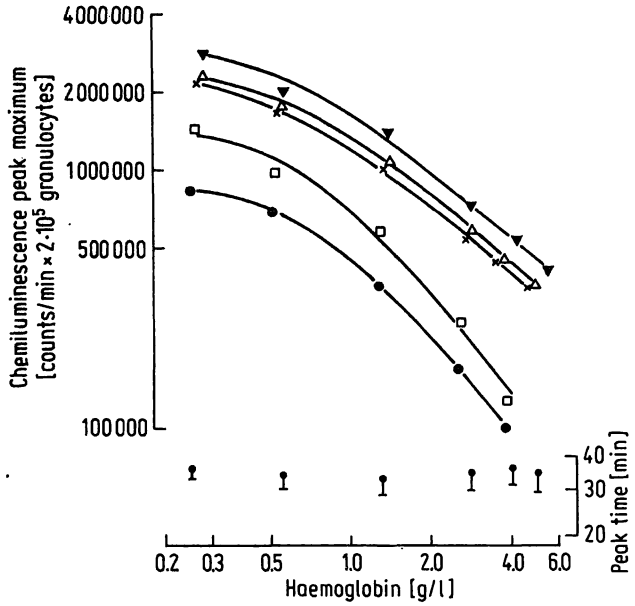



Fig. 1. Dependence of erythrocyte concentration (haemoglobin, g/l assay) on chemiluminescence peak maximum (counts/min × $2 \cdot 10^5$ granulocytes) and on peak time (min) in whole blood. Various volumes of erythrocytes were balanced with Minimal Essential Medium. Luminol (10 µl; 0.4 mmol/l final assay), non-opsonized zymosan (20 µl; 3.5 g/l final assay), granulocyte number ($0.5 \cdot 10^5$), plasma volume (20 µl) and final test volume of 570 µl are constant. Peak maximum values are plotted separately for each of the 5 probands. Peak times are plotted as means ± SD.

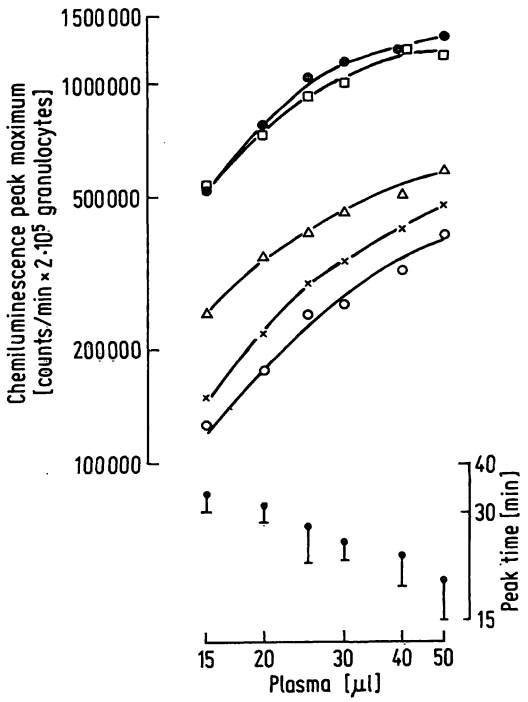



Fig. 2. Dependence of plasma volume fraction on chemiluminescence peak maximum (counts/min × $2 \cdot 10^5$ granulocytes) and on peak time (min) in whole blood. Various volumes of plasma (from 15 µl to 50 µl) were balanced with Minimal Essential Medium. Luminol (10 µl; 0.4 mmol/l final assay), non-opsonized zymosan (20 µl; 3.5 g/l final assay), granulocyte number ($0.5 \cdot 10^5$), erythrocyte volume (20 µl) and final test volume of 570 µl are constant. Peak maximum values are plotted separately for each of the 5 probands. Peak times are plotted as means ± SD.

With increasing numbers of red blood cells, peak maximum values are drastically reduced, whereas time peak values remain unchanged.

*Plasma volume*

On the other hand, if the numbers of erythrocytes and neutrophils are kept constant in an increasing plasma volume, the peak maximum increases and the peak time values become shorter (fig. 2).

*Plasma volume and erythrocytes*

Different combinations of plasma and red blood cells in a given constant volume of 40 µl with a constant number ($0.5 \times 10^5$) of neutrophils confirm both previous experiments. A shift to a higher volume fraction of erythrocytes against a lower volume fraction of plasma diminishes peak maximum counts and lengthens peak time (fig. 3).

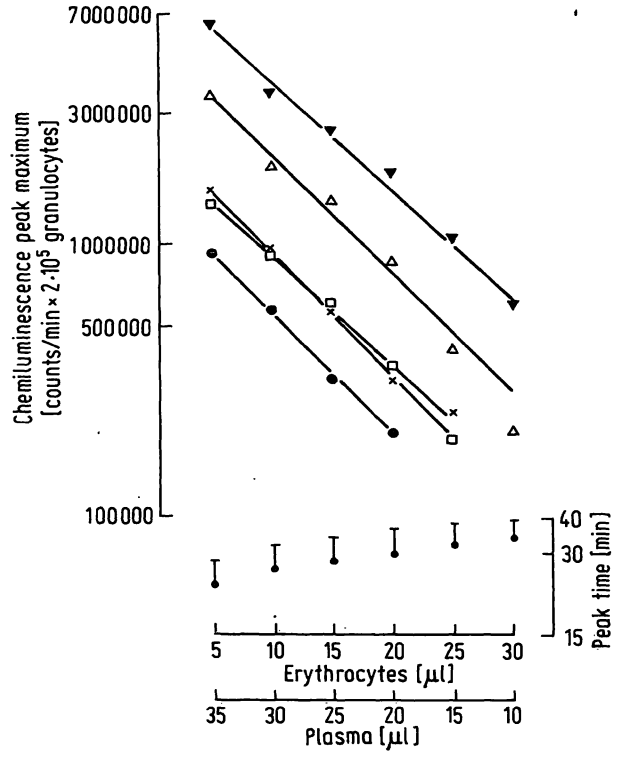



Fig. 3. Dependence of different ratios of plasma volume vs. erythrocytes on chemiluminescence peak maximum (counts/min × $2 \cdot 10^5$ granulocytes) and on peak time (min) in artificial whole blood. In a constant volume of 40 µl erythrocytes and plasma volume varied from 5 µl erythrocytes plus 35 µl plasma to 30 µl erythrocytes plus 10 µl plasma. Luminol (10 µl; 0.4 mmol/l final assay), non-opsonized zymosan (20 µl; 3.5 g/l final assay), granulocyte number ($0.5 \cdot 10^5$) and final test volume of 570 µl are constant. Peak maximum values are plotted separately for each of the 5 probands. Peak times are plotted as means ± SD.

*Granulocytes*

Figure 4 demonstrates that a proportional dependency exists between whole blood peak maximum values and granulocyte number. It is therefore possible to calculate a standardized chemiluminescence peak maximum for any blood sample, based on a fixed number of granulocytes. Individual peak time values are also unchanged (not shown). As each of the five probands has a different original whole blood granulocyte number to which additional granulocytes were added, a comprehensive graphical representation of peak time mean values is not possible.

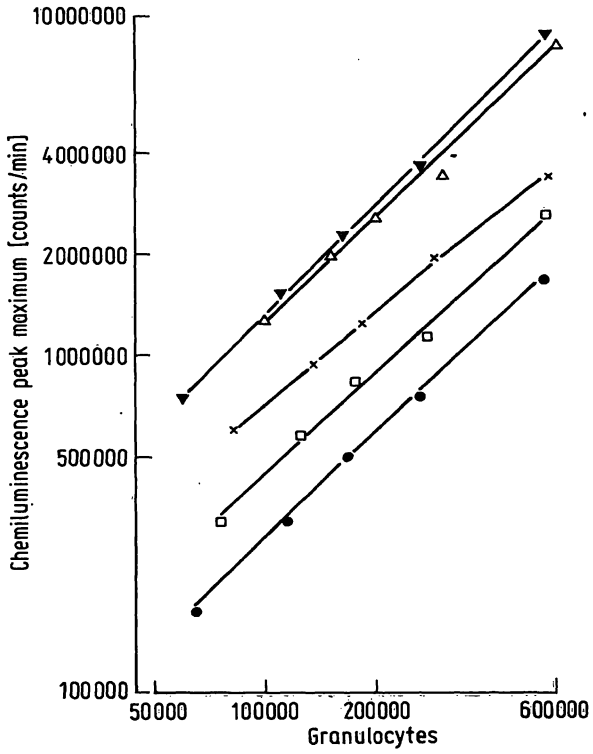


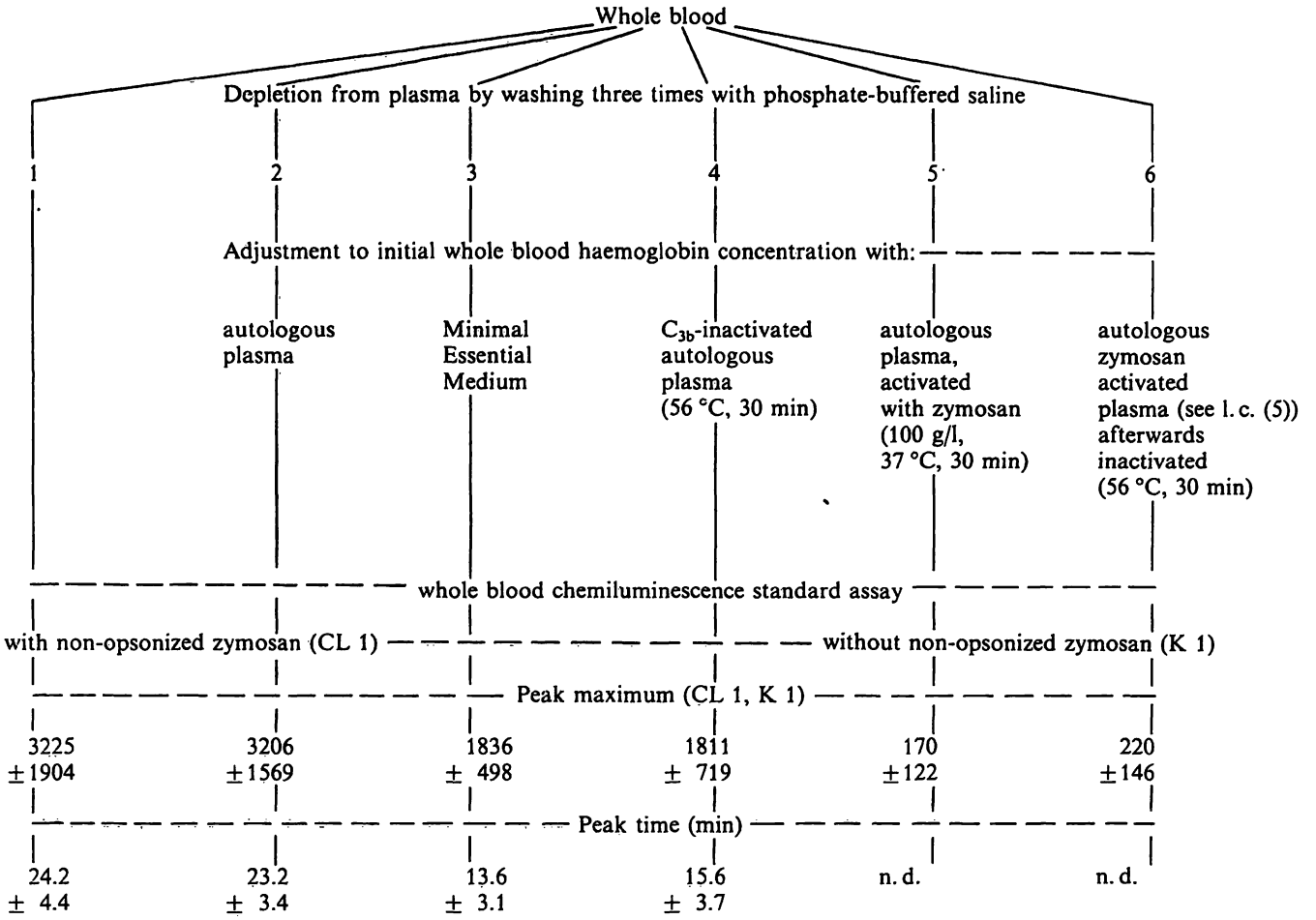

Fig. 4. Dependence of granulocyte number on chemiluminescence peak maximum (counts/min) in whole blood. Increasing granulocyte numbers (up to $500 \cdot 10^3$ cells) were added. Luminol (10 µl; 0.4 mmol/l final assay), non-opsonized zymosan (20 µl; 0.4 mmol/l final assay) and final test volume of 570 µl are constant. The values are plotted separately for each of the 5 probands.

*Plasma composition*

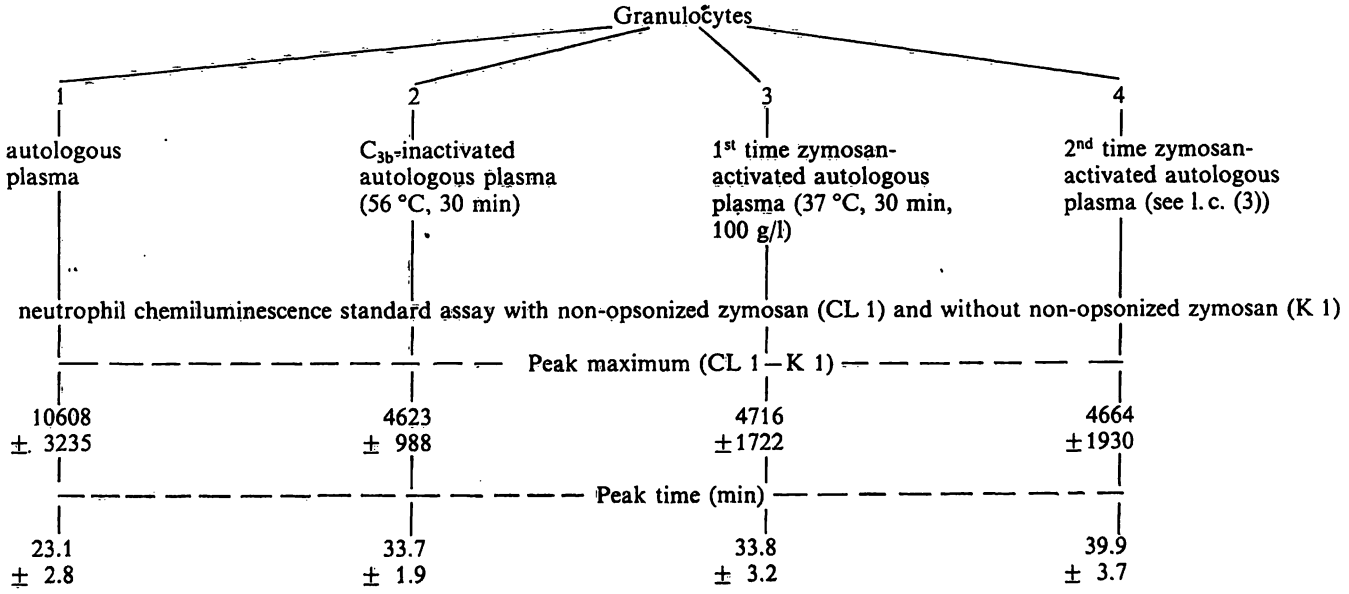
Washing of whole blood and readjustment to its initial haemoglobin concentration with autologous plasma does not alter the overall chemiluminescence response (tab. 1). On the other hand, substitution of plasma by buffer solution, by complement $C_{3b}$-inactivated plasma (30 min 56 °C) or by complement $C_{3b}$-desorbed plasma (100 mg zymosan per ml plasma, 30 min 37 °C) reduced the chemiluminescence response and shortened peak time values. Complement fraction $C_{5a}$ alone without non-opsonized zymosan, is therefore not a potent stimulus for the

chemiluminescence response. These results contradict those obtained for peak time values using isolated granulocytes (tab. 2). Complement $C_{3b}$ inactivation in the plasma fraction or its depletion from plasma by zymosan desorption results in diminished chemiluminescence but lengthens peak time.

Tab. 1. Chemiluminescence in untreated whole blood and in artificial whole blood whose plasma component has been replaced or treated in various ways as shown in the scheme. Peak maximum (CL 1, K 1; $10^3$ counts/min × 2 × $10^5$ granulocytes) and peak time (min) are given as mean ± SD; n = 11. n.d.: no clear cut peak time.




Tab. 2. Chemiluminescence in isolated granulocytes with the addition of plasma treated in various ways as shown in the scheme. Peak maximum (CL 1, K 1; $10^3$ counts/min × 0.5 × $10^5$ granulocytes) and peak time (min) are given as mean ± SD; n = 6.



*Normal values*

Normal values for chemiluminescence in whole blood and isolated phagocytes are given in tab. 3. Peak maximum values for granulocytes in whole blood are only about 0.05 of those for isolated neutrophils, based on the standardized phagocyte number.

Among isolated blood cells the neutrophils and eosinophils have an identical chemiluminescence pattern. Peak maximum (CL 1) values for monocytes, however, are only 0.3 of those for neutrophils and eosinophils. The peak maximum values (CL 2) for all three cell types are high and it is worth noting that this is the only non-opsonized zymosan triggered response without any opsonin.

## Superoxide dismutase, myeloperoxidase and catalase

Addition of superoxide dismutase (tab. 4) to either whole blood or neutrophil assays diminished the chemiluminescence response; myeloperoxidase increased the response, whereas catalase addition resulted in an increased response in the whole blood assay but

Tab. 3. Normal values for chemiluminescence in a) whole blood and b) isolated blood cells.
Whole blood: n = 81; 50 males, 31 females
Neutrophils: n = 54; 32 males, 22 females
Eosinophils: n = 8; 6 males, 2 females
Monocytes: n = 13; 11 males, 2 females
All values as mean ± standard deviation.

| | | | |
|---|---|---|---|
| a) Whole blood | | | |
| Peak maximum (CL 1) ($10^3$ counts/min × 2 × $10^5$ granulocytes) | 1627 ± 865 | | |
| Peak time (min) | 25.7 ± 4.5 | | |
| Peak maximum (K 1) ($10^3$ counts/min × 2 × $10^5$ granulocytes) | 22.5 ± 8.0 | | |
| b. Isolated blood cells | Neutrophils | Eosinophils | Monocytes |
| Peak maximum (CL 1) ($10^3$ counts/min × 0.5 × $10^5$ cells) | 8525 ± 2634 | 8850 ± 1174 | 3262 ± 1698 |
| Peak time (min) | 33.0 ± 7.3 | 32.1 ± 5.2 | 21.1 ± 7.2 |
| Peak maximum (CL 2) ($10^3$ counts/min × 0.5 × $10^5$ cells) | 5363 ± 1486 | 5110 ± 1883 | 2560 ± 834 |
| Peak time (min) | 48.3 ± 2.5 | 41.9 ± 5.6 | 18.5 ± 6.4 |
| Blank K 1 ($10^3$ counts/min × 0.5 × $10^5$ cells) | 104 ± 56 | 263 ± 89 | 405 ± 114 |
| Blank K 2 ($10^3$ counts/min × 0.5 × $10^5$ cells) | 171 ± 81 | 230 ± 64 | 149 ± 55 |

Tab. 4. Influence of superoxide dismutase, myeloperoxidase and catalase on zymosan-induced (non-opsonized and opsonized) chemiluminescence in whole blood and isolated neutrophils. Results are expressed as quotients of chemiluminescence peak maximum values (CL 1 − K 1) in the presence and in the absence of these enzymes. Final assay concentration of enzymes in brackets. Whole blood (20 µl) or isolated neutrophils ($0.5 \times 10^5$) were preincubated at 37 °C for 5 min with the enzymes before zymosan was added. $\bar{x}$ ± SD of five to six paired experiments; n. d.: not determined.

| | Whole blood | | 'Whole blood' | Neutrophils | | |
|---|---|---|---|---|---|---|
| Zymosan<br>Plasma | non-opsonized<br>inherent | opsonized<br>inherent | opsonized<br>depleted | non-opsonized<br>added (20 µl) | opsonized<br>added (20 µl) | opsonized<br>– |
| Superoxide dismutase (400 kU/l) | 0.70 ±0.11 | 0.74 ±0.07 | 0.73 ±0.06 | 0.58 ±0.06 | 0.59 ±0.08 | 0.70 ±0.06 |
| Myeloperoxidase (200 U/l) | 1.13 ±0.08 | n. d. | n. d. | 1.23 ±0.08 | 1.24 ±0.19 | 1.35 ±0.19 |
| Catalase (4800 kU/l) | 1.37 ±0.18 | 1.32 ±0.18 | 1.39 ±0.15 | 0.49 ±0.06 | 0.58 ±0.05 | 0.74 ±0.11 |

a reduced response for isolated neutrophils. To clarify the degree of contribution of naturally occurring plasma enzymes, stimulation with opsonized, as well as non-opsonized zymosan and the effect of addition or withdrawal of plasma were studied.

### Metabolic inhibitors and scavengers of reactive oxygen species

When phagocytes in whole blood or isolated neutrophils were subjected to the inhibitory effects of alanine (scavenger of HClO), mannitol and sodium benzoate (scavengers of hydroxyl radical) and histidine (scavenger of singlet oxygen) (4, 13), the most appreciable effect was observed for sodium benzoate. Azide, known to be an inhibitor of haem enzymes like catalase and myeloperoxidase (13), totally suppressed chemiluminescence, whereas cyanide, which additionally inhibits superoxide dismutase had only a moderate inhibitory effect (tab. 5).

Inhibitors of arachidonic acid metabolism are very powerful inhibitors of the chemiluminescence response. Nordihydroguaiaretic acid was used to inhibit the lipoxygenase pathway (4), indometacin to inhibit the cyclooxygenase pathway (4), and quinacrine to inhibit arachidonic acid production from membrane phospholipids, presumably by phospholipase $A_2$ inhibition (14).

When arachidonic acid, the substrate for both the lipoxygenase and the cyclooxygenase pathways was added, a small inhibitory effect was observed. Intracellular cAMP, which inhibits phosphoinositide turnover in human neutrophils (15), was raised by preincubation either with dibutyryl cAMP and/or theophylline (15), with prostaglandins or with NaF (4). It was found that all agents effectively suppressed the chemiluminescence response.

Inhibition of the ubiquitious calcium-binding protein calmodulin, using chlorpromazine (16), largely diminished chemiluminescence in both whole blood and isolated neutrophils.

### Receptor studies

N-acetylglucosamine and EDTA have been reported to inhibit complement receptor type three mediated unspecific binding to non-opsonized zymosan and to $C_{3b}$ fragment coated zymosan and to have no detectable effect on complement receptor type one or Fc-receptors (5). In our chemiluminescence assay EDTA blocked almost every response, irrespective of the particular opsonin added to the isolated neutrophils or the naturally occurring and generating opsonins in whole blood.

N-acetylglucosamine also effectively reduced the chemiluminescence response. In contrast to the above mentioned specificity, however, it also affects the Fc-receptor mediated response, as becomes evident from the results using immunoglobulin G as the specific opsonin.

Anti-immunoglobulin G had no effect on isolated neutrophils when plasma (i.e. mainly IgG and nascent $C_{3b}$) served as opsonins. This contrasts markedly with the response found in the whole blood assay.

Tab. 5. Inhibition of non-opsonized zymosan-induced chemiluminescence of phagocytes in whole blood and of isolated polymorphonuclear leukocytes by metabolic inhibitors and by scavengers of activated oxygen species. Inhibition is expressed as the quotient of chemiluminescence peak maximum values (CL 1 − K 1) in the presence and in the absence of inhibitors or scavengers. Final assay concentration of inhibitors and scavengers in brackets. Whole blood (20 µl) or isolated polymorphonuclear cells ($0.5 \times 10^5$) were preincubated at 37 °C for 10 min with inhibitors or scavengers before non-opsonized zymosan was added. $\bar{x} \pm$ SD of four to five paired experiments.

| Addition | Whole blood | Neutrophils |
|---|---|---|
| Alanine (10.5 mmol/l) | 0.82 ± 0.06 | 0.85 ± 0.05 |
| Mannitol (40 mmol/l) | 0.67 ± 0.15 | 0.86 ± 0.02 |
| Sodium benzoate (10.5 mmol/l) | 0.13 ± 0.02 | 0.28 ± 0.09 |
| Histidine (21 mmol/l) | 0.59 ± 0.07 | 0.73 ± 0.08 |
| Azide (1 mmol/l) | 0.03 ± 0.01 | 0.03 ± 0.01 |
| Cyanide (1 mmol/l) | 0.88 ± 0.07 | 0.83 ± 0.15 |
| Nordihydroguaiaretic acid (0.1 mmol/l) | 0.02 ± 0.001 | 0.05 ± 0.001 |
| Indometacin (1 mmol/l) | 0.02 ± 0.003 | 0.03 ± 0.004 |
| Quinacrine (1 mmol/l) | 0.01 ± 0.004 | 0.006 ± 0.002 |
| Arachidonic acid (0.05 mmol/l) | 0.90 ± 0.10 | 0.96 ± 0.08 |
| Dibutyryl cAMP (1 mmol/l) | 0.25 ± 0.05 | 0.19 ± 0.08 |
| Theophylline (2 mmol/l) | 0.28 ± 0.01 | 0.39 ± 0.02 |
| Dibutyryl cAMP (1 mmol/l) + theophylline (2 mmol/l) | 0.05 ± 0.01 | 0.02 ± 0.002 |
| Prostaglandin $E_1$ (0.03 mmol/l) | 0.08 ± 0.03 | 0.18 ± 0.04 |
| Prostaglandin $E_2$ (0.03 mmol/l) | 0.03 ± 0.01 | 0.08 ± 0.02 |
| NaF (20 mmol/l) | 0.006 ± 0.003 | 0.001 |
| Chlorpromazine (0.1 mmol/l) | 0.24 ± 0.09 | 0.28 ± 0.07 |

Anti-complement $C_3$, on the other hand, has a greater reducing effect in the neutrophil assay with plasma as the source of opsonins than in the whole blood assay. Anti-complement $C_{3b}$ is without any significant effect.

A variety of monoclonal antibodies, including OKM 1, recognize a cell surface molecule on phagocytes and it is suggested that this cell surface antigen is the $iC_{3b}$ receptor (5, 6). In the case of chemiluminescence, however, OKM 1 caused only slight inhibition in the whole blood assay and in the assay with isolated neutrophils with plasma as the opsonin source.

## Discussion

### Optimization of whole blood chemiluminescence; normal values for whole blood and isolated phagocytes

Dilutions of whole blood in the final assay mixture in reported methods for whole blood chemiluminescence range from 1 : 5 to 1 : 8000 (see l. c. (1, 2)). In our system blood is finally diluted 1 : 14. If quantitation of phagocyte-specific oxygenation activity is the sole objective of testing, relatively high dilutions of blood are needed. At a whole blood dilution of 1 : 8000 to peak maximum nearly equals that of isolated granulocytes (1). Photon yield is drastically reduced with increasing erythrocyte haemoglobin concentration (fig. 1). The peak maximum of whole blood phagocytes is only about 0.05 of that for an equivalent number of isolated granulocytes (tab. 3). There are three possible reasons for this phenomenon. Firstly, most of this decrease could be a physical consequence of photon absorption by haemoglobin (1). Secondly, erythrocytes serve as potent scavengers of activated oxygen species in vivo (17, 18) and in vitro (13). Thirdly, compared with isolated granulocytes, whole blood phagocytes possess distinctly fewer type 1 and type 3 complement receptors, both in the resting state, and after stimulation by activating stimuli (6), and they thus alter the activation profile. The importance of cell surface receptors on phagocytes and of plasma opsonins is discussed in detail below.

It is generally accepted, that lymphocytes and thrombocytes do not directly contribute to zymosan-induced chemiluminescence (11).

The relatively identical activation characteristic of granulocytes, i. e. neutrophils and eosinophils and the lower peak maximum values for stimulated monocytes (tab. 3) has, on the whole, been confirmed in comparative studies with eosinophils vs. neutrophils (19) and monocytes vs. neutrophils (20). Neutrophils, eosinophils and monocytes account for 0.87, 0.04 and 0.09, respectively, of the phagocytes in normal blood. According to the stimulation and distribution pattern of phagocytes, it is concluded that whole blood chemiluminescence is mainly the response of neutrophils. This is strengthened by the fact that proportional dependency exists between whole blood peak maximum values and granulocyte number (fig. 4). This is the basis for expressing whole blood chemiluminescence on the basis of a constant cell number, independent of other cellular and humoral implications.

### Addition of superoxide dismutase, myeloperoxidase, catalase and of metabolic inhibitors and scavengers of reactive oxygen species

Luminol-dependent chemiluminescence seems to be completely linked with a reaction mediated by the myeloperoxidase-$H_2O_2$-halide system, whereas native luminol-independent chemiluminescence is also related to a not yet elucidated $O_2$-mediated reaction (3, 21—23). These two obviously different mechanisms should be distinguished from one another, as well as the stimulation with distinct particulate or soluble agents. *Cheung* et al. (4), however, postulated that the lipoxygenase (and cyclooxygenase) pathway is the source of zymosan-induced and luminol-enhanced chemiluminescence.

It should be clearly stated that only the mechanism of zymosan-stimulated luminol-dependent chemiluminescence is under discussion here. That the reactive oxygen species alone are the source of chemiluminescence is contradicted by the following observations:

1. Superoxide dismutase and catalase cannot completely inhibit chemiluminescence, regardless of the quantity of inhibitors used (3, 4). We even find augmented chemiluminescence in whole blood upon addition of catalase (tab. 4). This must be due to some, until now unexplained, properties of the weak erythrocyte chemiluminescence response (24).

2. Previous studies have revealed that the excited oxygen species are principally capable of oxidizing luminol (23). Nevertheless, the magnitude of response is minimal and the time course very fast compared with zymosan-activated neutrophils (3, 4). This observation has led to the general comment that these oxidizing agents are not by themselves sufficient for light emission (4).

3. When neutrophils were subjected to the inhibitory effects of alanine (scavenger of hypochlorous acid), mannitol (scavenger of hydroxyl radical), histidine (scavenger of singlet oxygen), cyanide (inhibitor of

superoxide dismutase, myeloperoxidase, catalase) only slight or moderate inhibition was observed. Sodium benzoate (scavenger of hydroxyl radical) and azide (inhibitor of catalase and superoxide dismutase) are more powerful (tab. 5). In luminol-dependent chemiluminescence systems the effect of scavengers and inhibitors of reactive oxygen species is hard to evaluate. Scavenging, quenching and inhibitory effects overlap and contradictory results have been obtained. The relative importance of these oxygen species as the source of chemiluminescence has been reported very differently (3, 4, 23).

However, in addition to the importance of the myeloperoxidase-$H_2O_2$-halide hypothesis (3, 22), which is supported by our results from myeloperoxidase addition (tab. 4), it is evident from our inhibitor studies of arachidonic acid metabolism that this pathway plays a central role in the chemiluminescence phenomenon.

In the present study we find that established inhibitors of the cyclooxygenase (indometacin) and lipoxygenase pathway (nordihydroguaiaretic acid) almost totally inhibit zymosan-induced chemiluminescence in whole blood and isolated neutrophils. Both substances have been reported to inhibit chemiluminescence in a dose-dependent manner, the latter being stronger (4, 25, 26). From these experiments alone, however, it cannot be deduced whether light emissions is directly or indirectly attributed to the cyclooxygenase or lipoxygenase pathway.

However, arachidonic acid metabolism alone via the cyclooxygenase and/or lipoxygenase pathway is probably not the source of zymosan-induced chemiluminescence. This is supported by the observation that neutrophils from patients with chronic granulomatous disease do not respond with zymosan-induced luminol-enhanced chemiluminescence, although the cells are able to metabolize arachidonic acid but lack the membrane NADPH oxidase and do not produce oxygen species (24, 26). Nevertheless, there are several lines of evidence which support the notion that arachidonic acid metabolism has a pivotal role to play in chemiluminescence generation. Quinacrine, an inhibitor of arachidonic acid production from membrane phospholipids presumably by phospholipase $A_2$ inhibition (4), totally suppresses zymosan-induced chemiluminescence. Other inhibitors of arachidonic acid metabolism are reagents which, by a different mode of action, lead to elevated levels of cAMP, like theophylline and/or dibutyryl cAMP, prostaglandins and NaF (4, 15). The agents mainly affect neutrophil response by inhibiting the phosphoinositide cycle (4, 15). It has been reported that agonist-induced phosphoinositide splitting as well as resynthesis are reduced by procedures aimed at increasing intracellular cAMP (15). A third mode of action on the neutrophil response is obtained with a treatment aimed at modulating the calcium concentration by inhibiting the calcium-binding protein calmodulin with chlorpromazine. The action of chlorpromazine has been reported as inhibition of the activity of the calcium-dependent protein kinase C, phospholipase $A_2$ and the NADPH oxidase (16).

A possible sequence of events leading to the generation of chemiluminescence by the interaction between the NADPH oxidase system and arachidonic acid metabolism is schematically presented in fig. 5. It is based on the results of the present study and additionally inspired and supported by the reports of *Cheung* et al. (4), *Bianca* et al. (15), *Phillips* et al. (27), *Müller* et al. (28) and *Waite* (29). The zymosan-induced membrane pertubation leads to the activation of phospholipase C and phospholipase $A_2$ by a binding protein which, until now, has remained unidentified. A large proportion of arachidonic acid released is derived from phosphatidylinositol, mediated by a calcium-dependent phospholipase C mechanism, and from phosphatidylcholine, suggesting a calcium-dependent phospholipase $A_2$ mechanism that could also explain the release from phosphatidylinositol. In order for arachidonate to be derived from the phospholipase C pathway a lipase must act on the diacylglycerol. The products of phospholipase C action, diacylglycerol and inositol trisphosphate have important actions without directly entering the arachidonate cascade. The latter is responsible for the mobilization of calcium from intracellular stores and the opening of plasma membrane calcium channels. Diacylglycerol activates protein kinase C. The activation is associated with a translocation of cytosol protein kinase C in the plasma membrane which, in turn, activates NADPH oxidase by phosphorylation of some component of the oxidase. Decrease of diacylglycerol, brought about by cAMP, suppresses NADPH oxidase activation i. e. it suppresses chemiluminescence under our experimental conditions. The oxygen free radicals and their intermediates act mainly as oxidants inside and outside the cell but are not sufficient for light emission. Arachidonic acid is oxidized via the cyclooxygenase and lipoxygenase pathway to prostaglandins, thromboxanes and hydroperoxyeicosatetraenoic acids and leukotrienes, respectively. These pathways are also insufficient for light emission. Although the mechanism of interaction between the NADPH oxidase system and arachidonic acid metabolism is not yet understood, it clearly multiplies the response enormously.

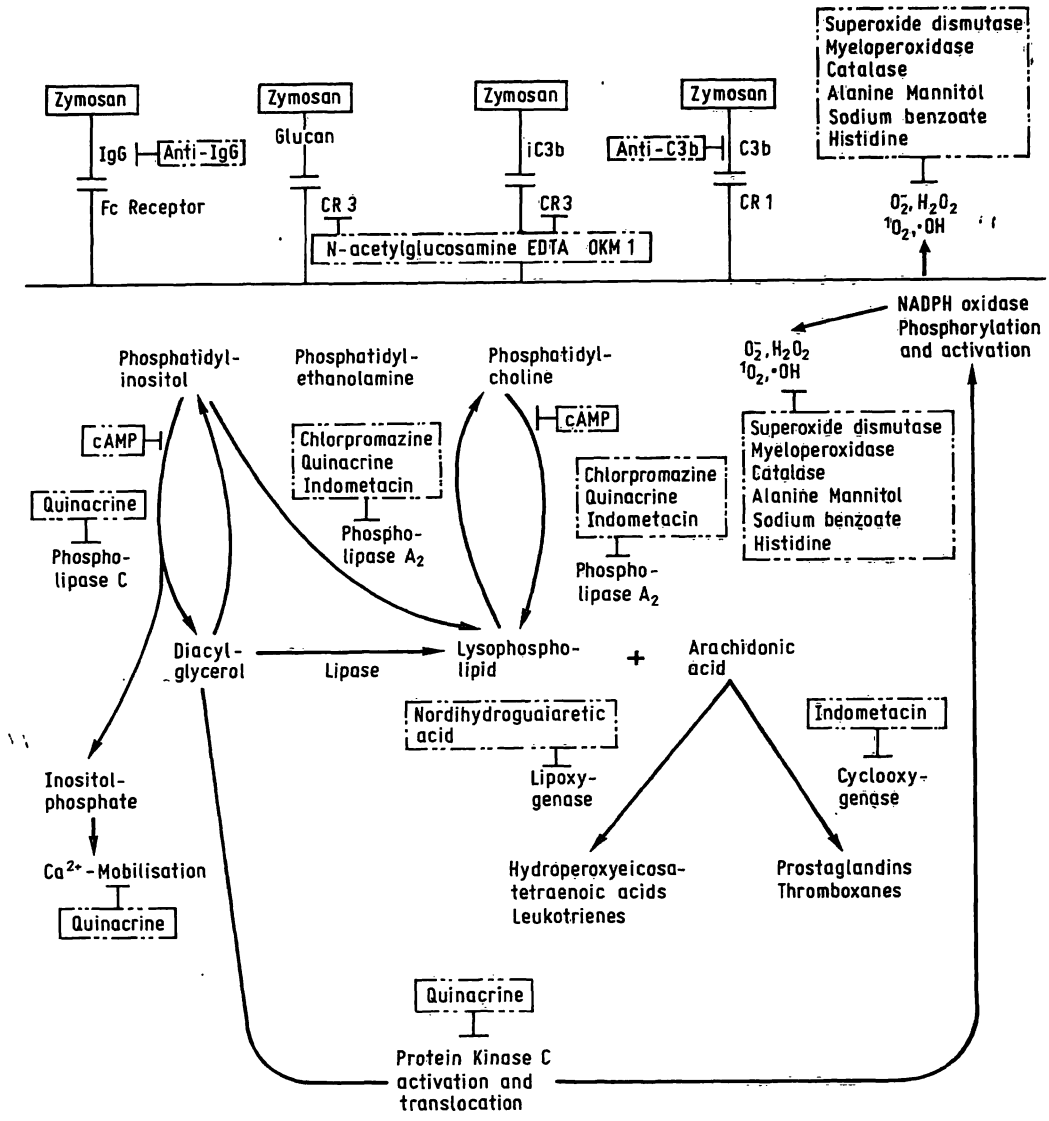



Fig. 5. Diagrammatic illustration of processes responsible for the generation of zymosan-induced and luminol-enhanced chemiluminescence. Scavengers and metabolic inhibitors are specified with their mode of action. For further details see text.

## Opsonins, receptors

Two main observations are relevant here:

1. Human blood phagocytes respond with chemiluminescence on interaction with zymosan and luminol in the absence of opsonins, and
2. the chemiluminescence is more evident if zymosan particles become coated with fragments of the third component of complement. These two aspects will be discussed separately.

### 1. Interaction without opsonins

Contradictory results have been reported concerning the need for opsonins as stimulation parameters of phagocytes (7). Our results on chemiluminescence, however, clearly reveal (tab. 3) that neutrophils, eosinophils and monocytes can all interact with zymosan in the absence of opsonins. This chemiluminescence response (CL 2) is 0.6 to 0.8 of that observed in the presence of plasma-derived opsonins (CL 1). *Schopf* at al. (20) found similar proportions for neutrophils and monocytes in response to unspecific as well as opsonin-mediated chemiluminescence. Very recently, *Williams* et al. (30) have shown that the major polysaccharide component of zymosan responsible for the chemiluminescence response of granulocytes is glucan.

### 2. Interaction in the presence of opsonins

Human blood phagocytes possess on their surface distinct phagocytic receptors for the Fc portion of immunoglobulin G as well as receptors for surface-bound cleavage products of the third component of complement. These include the complement receptor type 1 (CR 1) and type 3 (CR 3), recognizing particle-bound $C_{3b}$ and $iC_{3b}$, respectively, which are generated

by zymosan particles via the alternative pathway (5–7). We will discuss the role of opsonins and the role of cell surface receptors separately.

*Opsonins*

Results from depletion and omission of plasma, and physical treatment which affects the third complement fraction and/or immunoglobulin G permit the conclusion that $C_3$-derived fragments on zymosan are the major opsonic component; immunoglobulin G is of minor importance. The complement-independent chemiluminescence response is triggered by the opsonin-independent glucan polysaccharide component of zymosan.

The results of chemical treatment with specific antibodies directed against $C_3$, $C_{3b}$ and immunoglobulin G, respectively have to be differentiated (tab. 6). The results with anti-immunoglobulin are critical for several reasons. Firstly, even highly purified immunoglobulin G with a negligible number of polymers induces a dose-dependent stimulation of neutrophils as measured by chemiluminescence (31). Secondly, soluble immunocomplexes are potent activators of the chemiluminescence response (32). We also observed that the chemiluminescence with immunoglobulin G alone, without zymosan, is 0.3 of that measured for the immunoglobulin G-opsonized zymosan-induced response (absolute values from tab. 6, not shown). Naturally occurring immunoglobulin G from plasma, however, in the same concentration range, obviously does not respond in this way (tab. 1, tab. 3). This qualifies the values on anti-immunoglobulin G from tab. 6. The inhibition studies with complement antibodies (tab. 6) indicate that $iC_{3b}$ fixed on zymosan promotes the main chemiluminescence response. $C_{3b}$ is of minor importance.

*Cell surface receptor*

It has been suggested that complement receptor type 3 binds to both fixed $iC_{3b}$ and non-opsonized zymosan i.e. the glucan component, and should be inhibited by either EDTA or N-acetylglucosamine (5, 7). EDTA blocks the chemiluminescence response in whole blood and from isolated neutrophils more completely than N-acetylglucosamine (tab. 6). The effect of both inhibitors with immunoglobulin G as opsonin is essentially not mediated by an effect on Fc-receptors, because the decrease is mainly in the non-opsonized zymosan-mediated reaction. The latter has already been discussed to be dependent on complement receptor type 3 binding. These results again confirm our conclusion regarding the secondary importance of immunoglobulin G as opsonin for zymosan-mediated chemiluminescence.

Several monoclonal antibodies including OKM 1 recognize a cell surface molecule on neutrophils, and it is suggested that the antigen recognized by these antibodies is the $iC_{3b}$ receptor CR 3 (5–7), although blocking of the chemiluminescence response with OKM 1 was not very pronounced in our experiments (tab. 6).

With regard to this, there appears to be a contradiction in the finding that in whole blood the peak time is shortened by treatments affecting complement factor type 3 (tab. 1), whereas it lengthens in the assay with isolated cells (tab. 2, tab. 3); a common feature was the reduction of peak maximum values.

It has been shown that granulocytes in whole unseparated blood express relatively few receptors for $C_{3b}$ (CR 1) or $iC_{3b}$ (CR 3) but substantially more on isolated granulocytes. Isolated cells continue to respond to stimuli by further increasing membrane expression

Tab. 6. Influence on neutrophil and whole blood chemiluminescence by chemicals which react with surface membrane receptors on phagocytes or with opsonins. Results are expressed as quotients of chemiluminescence peak maximum values (CL 1 – K 1, CL 2 – K 2) in the presence and in the absence of these chemicals. Final assay concentration for N-acetylglucosamine, EDTA and OKM 1 were 200 mmol/l, 3 mmol/l and 5 mg/l, respectively. Antisera were diluted according to the titre. Neutrophils ($0.5 \times 10^5$) or whole blood (40 µl) were incubated with the chemicals according to the standard pipetting scheme in the material and methods section. Chemiluminescence was initiated with non-opsonized zymosan. In addition to the standard procedure with autologous plasma (20 µl), immunoglobulin G (20 µl of a 11 g/l solution) serves as specific opsonin and phosphate-buffered saline as control without any opsonin. $\bar{x} \pm$ SD of six to eight paired experiments.

| | Neutrophils | | | Whole blood |
|---|---|---|---|---|
| | Plasma | Immunoglobulin G | Saline | |
| OKM 1 | 0.87 ± 0.07 | 1.04 ± 0.05 | 1.13 ± 0.08 | 0.89 ± 0.05 |
| N-acetylglucosamine | 0.24 ± 0.07 | 0.56 ± 0.19 | 0.20 ± 0.09 | 0.05 ± 0.03 |
| EDTA | 0.02 ± 0.01 | 0.08 ± 0.03 | 0.04 ± 0.02 | 0.04 ± 0.02 |
| Anti-immunoglobulin G | 1.01 ± 0.13 | 0.79 ± 0.06 | 0.99 ± 0.05 | 0.56 ± 0.23 |
| Anti-complement $C_3$ | 0.55 ± 0.25 | 0.95 ± 0.07 | 1.04 ± 0.11 | 0.89 ± 0.12 |
| Anti-immunoglobulin G + Anti-complement $C_3$ | 0.86 ± 0.15 | 0.85 ± 0.13 | 1.03 ± 0.09 | 0.41 ± 0.22 |
| Anti-complement $C_{3b}$ | 1.02 ± 0.06 | 0.96 ± 0.05 | 1.10 ± 0.08 | 0.89 ± 0.07 |

of these receptors to a greater extent than whole blood granulocytes. These additional receptors are likely to be translocated from an intracellular pool to the surface (6), thus lengthening the peak time value (tab. 1 vs. tab. 2, tab. 3). It is advisible, therefore, to draw only very cautious conclusions about whole blood or even in vivo conditions from observations made on isolated cells.

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## References

1. Allen, R. C., Mead, M. E. & Kelly, J. L. (1985) In: CRC handbook of methods for oxygen radical research (Greenwald, R. A., ed.), pp. 343–351, CRC Press, Boca Raton.
2. Bruchelt, G. & Schmidt, K. H. (1984) J. Clin. Chem. Clin. Biochem. *22*, 1–13.
3. De Chatelet, L. R., Long, G. D., Shirley, P. S., Bass, D. A., Thomas, M. J., Henderson, F. W. & Cohen, M. S. (1982) J. Immunol. *129*, 1589–1593.
4. Cheung, K., Archibald, A. C. & Robinson, M. F. (1983) J. Immunol. *130*, 2324–2329.
5. Ross, G. D. & Medof, M. E. (1985) Adv. Immunol. *37*, 217–367.
6. O'Shea, J. J., Brown, E. J., Seligman, B. E., Metcalf, J. A., Frank, M. M. & Gallin, J. I. (1985) J. Immunol. *134*, 2580–2587.
7. Ross, G. D., Cain, J. A. & Lachmann, P. J. (1985) J. Immunol. *134*, 3307–3315.
8. Heberer, M., Ernst, M., Dürig, M., Allgöwer, M. & Fischer, H. (1982) Klin. Wochenschr. *60*, 1443–1448.
9. Van Kampen, E. J. & Zijlstra, W. G. (1961) Clin. Chim. Acta *6*, 538–544.
10. Lindena, J., Wittenberg, H., Diederichs, F. & Trautschold, I. (1986) J. Clin. Chem. Clin. Biochem. *24*, 49–59.
11. Schopf, R. E., Hammann, K. P., Scheiner, O., Lemmel, E.-M. & Dierich, M. P. (1982) Immunology *46*, 307–312.
12. Roberts, R. L. & Gallin, J. I. (1985) Blood *65*, 433–440.
13. Test, S. T. & Weiss, S. J. (1984) J. Biol. Chem. *259*, 399–405.
14. Seeger, W., Suttorp, N., Schmidt, F. & Neuhof, H. (1986) Am. Rev. Respir. Dis. *133*, 1029–1036.
15. Della Bianca, V., De Togni, P., Grzeskowiak, M., Vicentini, L. M. & Di Virgilio, F. (1986) Biochim. Biophys. Acta *886*, 441–447.
16. Naccache, P. H. (1985) In: Calmodulin antagonists and cellular physiology (Hidaka, H. & Hartshorne, D. J., eds.), pp. 149–159, Academic Press, Orlando.
17. Toth, K. M., Clifford, D. P., Berger, E. M., White, C. W. & Repine, J. E. (1984) J. Clin. Invest. *74*, 292–295.
18. Agar, N. S., Sadrzadeh, S. M. H., Hallaway, P. E. & Eaton, J. W. (1986) J. Clin. Invest. *77*, 319–321.
19. Shult, P. A., Graziano, F. M., Wallow, J. H. & Busse, W. W. (1985) J. Lab. Clin. Med. *106*, 638–645.
20. Schopf, R. E., Straussfeld, E. & Morsches, B. (1985) Z. Hautkr. *60*, 797–798.
21. Roschger, P., Graninger, W. & Klima, H. (1984) Biochem. Biophys. Res. Commun. *123*, 1047–1053.
22. Dahlgren, C. & Briheim, G. (1985) Photochem. Photobiol. *41*, 605–610.
23. Brestel, E. P. (1983) Biochem. Biophys. Res. Commun. *126*, 482–488.
24. Peerless, A. G. & Stiehm, E. R. (1986) Clin. Immunol. Immunopathol. *38*, 1–12.
25. Yocum, D. E., Hempel, S. & Busse, W. W. (1984) J. Immunopharmacol. *6*, 237–255.
26. Henricks, P. A., van der Tol, M. E., van Kats-Renaud, J. H., Nijkamp, F. P. & Verhoef, J. (1984) Biochim. Biophys. Acta *801*, 206–214.
27. Phillips, W. A., Mossmann, H. & Ferber, E. (1986) J. Leuk. Biol. *39*, 267–284.
28. Mueller, H. W., O'Flaherty, J. T., Greene, D. G., Samuel, M. P. & Wykle, R. L. (1984) J. Lipid. Res. *25*, 383–388.
29. Waite, M. (1985) J. Lipid Res. *26*, 1379–1388.
30. Williams, J. D., Topley, N. Alobaidi, H. M. & Harber, M. J. (1986) Immunology *58*, 117–124.
31. Koch, C., Vaterius, N. H. & Andersen, V. (1984) Acta Pathol. Microbiol. Immunol. Scand. 92, 161–165.
32. Cheung, K., Archibald, A. C. & Robinson, M. F. (1984) Aust. J. Exp. Biol. Med. Sci. *62*, 403–419.

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